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60/556474

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

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☐ *Additional inventors are being named on the ____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

GELDANAMYCIN AND DERIVATIVES INHIBIT CANCER INVASION AND IDENTIFY NOVEL TARGETS

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 13 ☐ CD(s), Number

☒ Drawing(s) *Number of Sheets* 5 ☐ Other (specify) Return Postcard

☒ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

☒ Applicant claims small entity status. See 37 CFR 1.27.

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: ____.

Respectfully submitted,

SIGNATURE _____

TYPED or PRINTED NAME Shmuel Livnat

TELEPHONE 202-344-4000

Date March 26 2004

REGISTRATION NO. *(if appropriate)* 33,949

Docket Number: 38345-201708

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. and is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. SEND TO: **Box Provisional Application**, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

#534793

VENABLE ATTORNEYS AT LAW

Geldanamycin and Derivatives Inhibit Cancer Invasion and Identify Novel Targets

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention in the field of cancer pharmacology is directed to novel agents, geldanamycin derivatives, that show inhibitory activity at femtomolar concentrations.

Description of the Background Art

Geldanamycin is an ansamycin natural product drug. GA and its derivatives have been shown to inhibit the growth of human carcinoma cells in a dose-dependent manner and have antitumor activity in breast, melanoma, and ovarian mouse xenograft models in athymic nude mice (6). These compounds work by reducing the intracellular concentration of a large number of tyrosine and serine kinase oncogene products, including Her2, Met, Raf, cdk4, and Akt (1,7,8). A recent study with prostate cancer cells found that GA stimulated degradation of HIf-1α, suggesting that the drug may also inhibit vasculogenesis (9-11). GA drugs at nanomolar levels inhibit the function of the molecular chaperone HSP90, preventing proper folding of oncoproteins, thus promoting their destabilization and leading to inhibition of tumor growth (1, 12-14).

GA is not used clinically due to solubility and toxicity limitations, but 17-AAG (NSC.330507), a GA derivative that shows tumor inhibitory activity with less toxicity (15), is being evaluated in phase I–II clinical trials (16, 17). Another GA derivative in preclinical evaluation is 17-DMAG, which has greater water solubility and is available for oral delivery. The bioavailability of 17-DMAG is essentially 100% when given i.p., about twice that of orally delivered 17-AAG (18). 17-AG, a metabolite of 17-AAG, has equivalent biological activity as determined by the ability to decrease $p185^{erbB2}$ and is under development as a potential therapeutic (19). In breast cancer, both GA and 17-AAG can sensitize the cells to Taxol and doxorubicin-mediated apoptosis (20).

The SF/HGF-Met signal pathway has been widely accepted as playing an essential role in tumor proliferation, invasion, and metastasis. Met and its ligand, HGF/SF, contribute to tumorigenesis and metastasis in all types of solid tumors (21, 22), and the multiple activities of HGF/SF-Met signaling pathways leading to proliferation, invasion, angiogenesis, and cell

survival are well characterized (23-27). Previously, our lab discovered that GA and several derivatives inhibit urokinase-plasmin network at femtomolar concentrations only in MDCK cells.

Previous work from the present inventors' laboratory showed that only 4 out of over 30 geldanamycin (GA)-derived drugs provided by the NCI Anti-Neoplastic Drug Screen Program (NCI ADS) inhibited the activation of urokinase plasminogen activator (uPA)-plasmin by hepatocyte growth factor/scatter factor (HGF/SF) in MDCK cells at femtomolar concentrations (Ref. 1: Webb CP *et al.*, *Cancer Res.* 60: 342–3491). There drugs are referred to herein as "GafM" drugs versus drugs of the GA family drugs that show activity in the nanomolar range (referred to as "GanM" drugs.

SUMMARY OF THE INVENTION

Some of the drugs listed in the publication by Webb *et al.* (*supra*) as supplied by the NCI-Ads were found not to be pure, leading to earlier incorrect interpretations. A study in collaboration with co-inventors Drs. David Wenkert and Yuehai Shen has resolved the problem of impurities in the original samples. The inventors synthesized the active GA derivatives. This is the first report that GA and certain derivatives have the same function in tumor cells through two independent experiments. By using the uPA assay as a method measuring uPA-plasmin network activity, it was found that GA and 17-AAG inhibit the network at femtomolar (fM) concentrations, as observed in MDCK cells (Figure 2). By contrast, in a basement membrane Matrigel® invasion assay, the number of GBM cells that penetrated through Matrigel® was increased almost 10-fold with HGF/SF treatment (100 ng/ml) relative to the control group; however, invasion was almost completely inhibited by GA at fM (Figure 3). Thus, these drugs are candidates for interfering with tumor cell invasion, and may be combined with surgery, conventional chemotherapy, or radiotherapy to prevent cancer cell invasion.

In parallel studies, the novel target of GAfM drugs was identified. Previously reported targets include HSP90, Grp94, and TRAP1 (tumor necrosis factor receptor-associated protein 1), and the therapeutic effects of the GA drug family are addressed mainly through HSP90α (5). By using GA-coated Affigel beads, we discovered VDAC1 protein as a novel target that can bind to GAfM. VDAC1 is a group of proteins forming the aqueous pore on the mitochondrial outer membrane that elicit the crosstalk between mitochondria and the rest of the cell.

Recently, VDAC has been implicated in the early steps of mitochondria-dependent apoptosis (28). The discovery of VDAC1 as a novel target of GAfM leads to the new applications of GA drugs directed against apoptosis mechanisms and more. The VDAC1 structure with GA can provide new drug design. New anticancer drugs can be designed and the implication of apoptosis on GAfM activity explored.

The present inventors have synthesized and purified all of the compounds listed herein. Testing of these compounds has led to the conclusion that the most active geldanamycin derivatives are those in which the 17-methoxy group of geldanamycin has been replaced by an amino or an alkylamino group. In the case of the latter, the alkyl substituent may be itself derivatized, but it needs to be short.

Such active geldanamycin derivatives are exemplified by 17-amino-17-demethoxygeldanamycin, 17-(*N*-allylamino)-17-demethoxygeldanamycin, 17-[2-(dimethylamino)-ethylamino]-17-demethoxygeldanamycin, and 17-(2-chloroethyl)amino-17-demethoxygeldanamycin. Derivatives that had longer 17-alkylamino substituents were not active; the latter include 17-[6-(acetylamino)hexylamino]-17-demethoxygeldanamycin and 17-[6-(*N*-succinyl)hexylamino]-17-demethoxygeldanamycin. Additionally the 17,18-derivatized compound 7'-bromogeldanoxazinone was found to not be active.

Modifications to the ansa ring of geldanamycin [that is, the carbon atoms of the large ring of geldanamycin excluding those of the benzoquinone ring, in positions 1 through 15 of geldanamycin] resulted in derivatives that have activity only in the nanomolar range. Examples of this are esters of the 11-hydroxyl group of geldanamycin.

The list of the drugs and their femtomolar activity against invasion in GBM cells is disclosed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

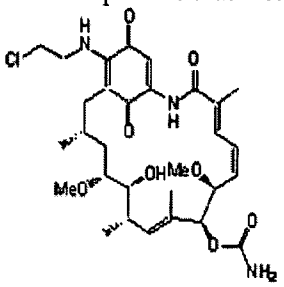
(see next section)

DESCRIPTION OF THE PREFERRED EMBODIMENTS

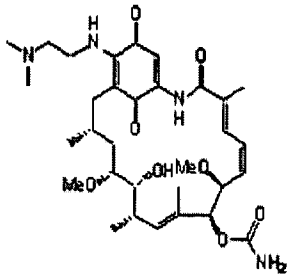
and

EXAMPLES

The compounds that were synthesized and tested are described below.



17-CEG



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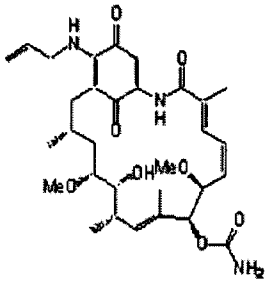

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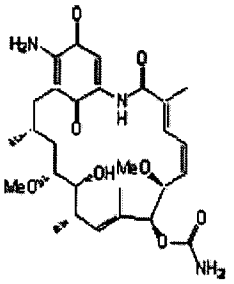
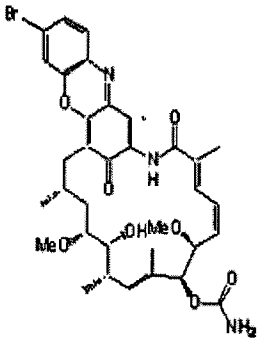
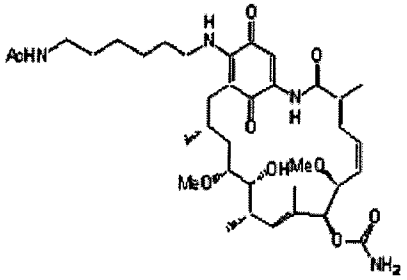


17-ADG



BGX



17-AHG

Geldanamycin derivatives: Names, molecular weights and activities

Entry	NSC* number	GVW code	YS code	Chemical Name (Abbr.)	Chemical name (Full)	M.W.	μ PA[1]
1	122750	GA		GA	geldanamycin	560.64	Yes
2	320877	F	YS1079	17-CEG	17-(2-**c**hloro**e**thyl)amino-17-demethoxy**g**eldanamycin	608.12	Yes [2]
3	683659	Q	YS1080	17-DMAG	17-(**dim**ethyl**a**minoethyl)amino-17-demethoxy**g**eldanamycin	616.75	Yes
4	330507	H	YS1057	17-AAG	17-**a**lyl**a**mino-17-demethoxy**g**eldanamycin	585.69	Yes
5	255109	V	YS1051	17-AG	17-**a**mino-17-demethoxy**g**eldanamycin	545.62	Yes
6	255105	B		BGX	7'-**b**romo-17-demethoxy**g**eldano**x**azone [3]	698.60	No
7	---		YS1025	17-AAHG[4]	17-(6-**a**cetyl**a**mino**h**exyl)amino-17-demethoxy**g**eldanamycin	686.84	No

[1] Yes = sample has activity at fM level; No = sample has activity only at nM level.

[2] Sample contaminated with ~10% geldanamycin.

[3] Reported name by K. L. Rinehart

[4] Affinity beads analog

* National Cancer Institute designation

As before, GA, 17-AAG, 17-CEG, and 17-AG display GAfM activity, but other compound in Table 1 are different than those in the original report (1) due to unexpected impurities in some of the original samples tested. The most active geldanamycin derivatives are those in which the 17-methoxy group of geldanamycin has been replaced by an amino or an alkylamino group. In the case of the latter, the alkyl substituent may be itself derivatized, but it needs to be short. Such active geldanamycin derivatives are exemplified by 17-amino-17-demethoxygeldanamycin, 17-(*N*-allylamino)-17-demethoxygeldanamycin, 17-[2-(dimethylamino)-ethylamino]-17-demethoxygeldanamycin, 17-(2-chloroethyl)amino-17-demethoxygeldanamycin, and 17-(2-fluoro)ethylamino-17-demethoxygeldanamycin. Derivatives that had longer 17-alkylamino substituents were not active. The latter included 17-[6-(acetylamino)hexylamino]-17-demethoxygeldanamycin and 17-[6-(*N*-succinyl)hexylamino]-17-demethoxygeldanamycin. Additionally the 17,18-derivatized compound 7′-bromogeldanoxazinone was found to not be active.

Table 1. High-activity GA derivatives (unpublished)

NSC No.	Abbrev.	Chemical Name
122750	GA	Geldanamycin
320877	17-CEG	17-(2-chloroethyl)amino-17-demethoxygeldanamycin
255109	17-AG	17-amino-17-demethoxy-geldanamycin
683659	17-DMAG	17-(dimethylaminoethyl)amino-17-demethoxygeldanamycin
	17-FEG	17-(2-fluoro)ethylamino-17-demethoxygeldanamycin
330507	17-AAG	17-allylamino-17-demethoxy-geldanamycin

Modifications to the ansa ring of geldanamycin (that is, the carbon atoms of the large ring of geldanamycin excluding those of the benzoquinone ring, or positions 1 through 15 of geldanamycin) resulted in derivatives that have activity only in the nanomolar range. Examples of this are esters of the 11-hydroxyl group of geldanamycin. The uPA assay shows that after HGF/SF treatment (10 ng/ml), uPA activity is up-regulated. When MDCK cells are treated with these drugs, however, the uPA activity can be inhibited down to the femtomolar

level, suggesting a potential use for these drugs as uPA inhibitors (Figure 1). Radicicol is a chemical which reportedly works the same as GA drugs, but it only shows nanomolar level activity against uPA-plasmin proteolysis in this assay and, therefore, it served as a negative control.

Discovery that human glioblastoma and sarcoma cells are also sensitive to GAfM.

Previous work from the present inventors' lab and others (2,3,4) has shown that GBM cell lines express Met and HGF/SF and display strong proliferative or invasive responses to HGF/SF (2). In screening several human GBM tumor cell lines using the uPA assay, one highly invasive line discovered to display GAfM inhibition of the uPA plasmin proteolysis network (Figure 2), as observed in MDCK cells (1) (Figure 1).

After treatment with HGF/SF (10 ng/ml), uPA activity was up-regulated (a control shows the activity is inhibited by HGF/SF-neutralizing mAb in GBM-DBTRG cells (Figure 1).

The striking result was that GA and 17-AAG inhibit HGF/SF-induced uPA activity at 10^{-13} M in DBTRG cells where Met expression is unaffected (Figure 3), while a GA derivative such as Macbecin II inhibited only in the nM range where Met expression is markedly inhibited (1) (Figure 2).

Human SK-LMS-1 leiomyosarcoma cells display similar, though somewhat less active, sensitivity to the GAfM drugs, illustrating that this is a more common target in tumor cells.

In addition to inhibition of the uPA-plasmin activity, the present inventors discovered that HGF/SF-mediated invasion through basement membrane (Matrigel®) was also blocked by the GAfM drugs at fM concentrations (Figure 3). The number of GBM cells that penetrated through Matrigel was significantly increased with HGF/SF treatment (100 ng/ml) when compared with the control group.

However, this invasion was dramatically inhibited by GAfM treatment down to a concentration of 10^{-15} M and 17-AAG worked as well (data not shown). This argues in favor of the present inventors conception that GAfM have promise for interfering with GBM tumor cell invasion *in vivo*.

Identification of Novel GA Targets

With the collaboration of co-inventors, Dr. David Wenkert and Yuehai Shen, novel GA targets were discovered in addition to the best known target, HSP90 (5).

GA was coated on the Affigel® beads and proteins that bound to GA could be recognized in pull-down experiments.

Results of the GA pull-down assays using DBTRG cells with control beads (lane 1) or GA beads (lanes 2-4) are shown in Figure 4.

In the indicated samples, 24 hours before cell lysis, GA or 17AAG was added to the cell cultures. This allowed us testing for GA bead competition in the pull-down assay.

In addition to HSP90, which is the previously reported GA target (5), the present inventors identified new potential GA target position candidates.

Candidate 2 is VDAC1 (voltage-dependent anion channel, also known as porin), a mitochondrial membrane protein which has been implicated in mitochondrial-related apoptosis in normal and tumor cells (28). The same pull-down assay was performed on MDCK cells and showed the same results.

Mitochondria enrichment for porin (Figure 5).

VDAC1 has been found on the membrane of mitochondria.

Therefore, mitochondria from MDCK cells in lysis buffer were subjected to to pull-down assays using GA affinity beads.

Lanes 1 and 2 are MDCK total cell lysates pulled down by control or GA affinity beads, as described above, followed by blotting with HSP90α and VDAC1 antibody acting as the negative and positive controls, respectively. Lanes 3–5 are samples generated from mitochondria enrichment using a Mitochondria Isolation Kit (Sigma Cat. Mito-Iso1).

Cells were cultured in 150x25-mm dishes. Ten dishes of cells were lysed with the buffer A (50 nM Hepes, pH 7.5, 1 M mannitol, 350 mM sucrose, 5 mM EGTA, and 2 mg/ml albumin solution), followed by sonication (pulsed 5x at 10 s per pulse). The samples were then centrifuged at 600 × *g* for 5 min to collect nuclei. The supernatant was removed and centrifuged at 1,1000 × *g* for 10 min to harvest the mitochondria. The supernatant, which contain cytosol was collected and pulled down by GA affinity beads; it showed mainly HSP90α in Lane 3.

The collected mitochondria were resuspended in lysis buffer and subjected to another cycle of centrifugation for further purification. Lane 4 is the supernatant after second centrifugation. The supernatant was again pulled down with GA affinity beads and showed less HSP90α.

Lane 5 is the purified mitochondrial fraction lysed with lysis buffer B (50 M Tris-HCL, pH7.5, 2 mM EDTA, 100 mM NaCl, 10 mM Na orthovanadate, 1% NP-40) with protease inhibitor cocktail tablet (Roch) and subjected to GA affinity beads. This fraction still contained a trace of HSP90α but mainly contained VDAC1. This experiment confirmed the hypothesis that VDAC1 directly binds to GA.

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All the references cited above are incorporated herein by reference in their entirety, whether specifically incorporated or not.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

ABSTRACT

GAfM drugs that block the uPA-plasmin network and inhibit GBM *in vitro* invasion have the possibility to be used to inhibit tumor invasion *in vivo* at femtomolar concentrations. An effective GA-based treatment would provide an option for treating invasive brain cancers and potentially could be combined with surgery, conventional chemotherapy, or radiotherapy with the purpose of preventing brain cancer invasion. The novel target of these femtomolar-level drugs may be a key regulatory molecule that promotes or prevents cell motility and invasion.

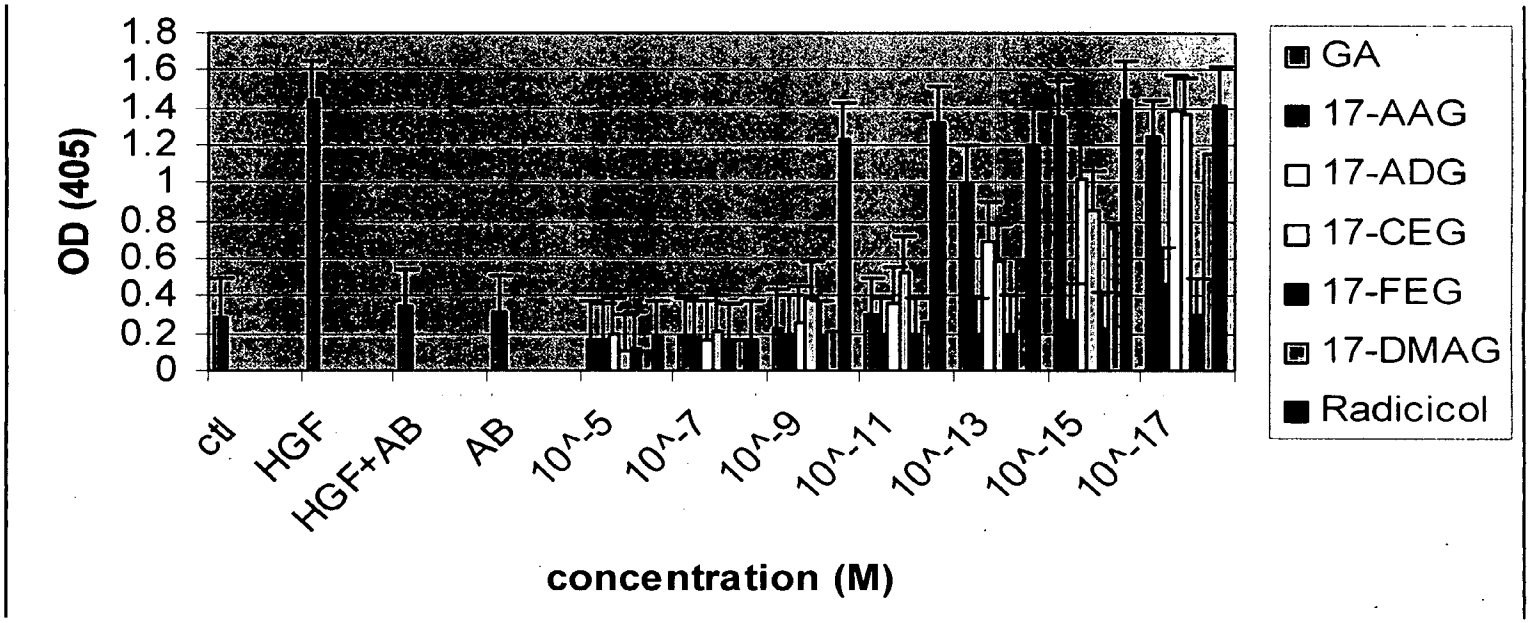


Fig. 1

uPA assay on MDCK cells

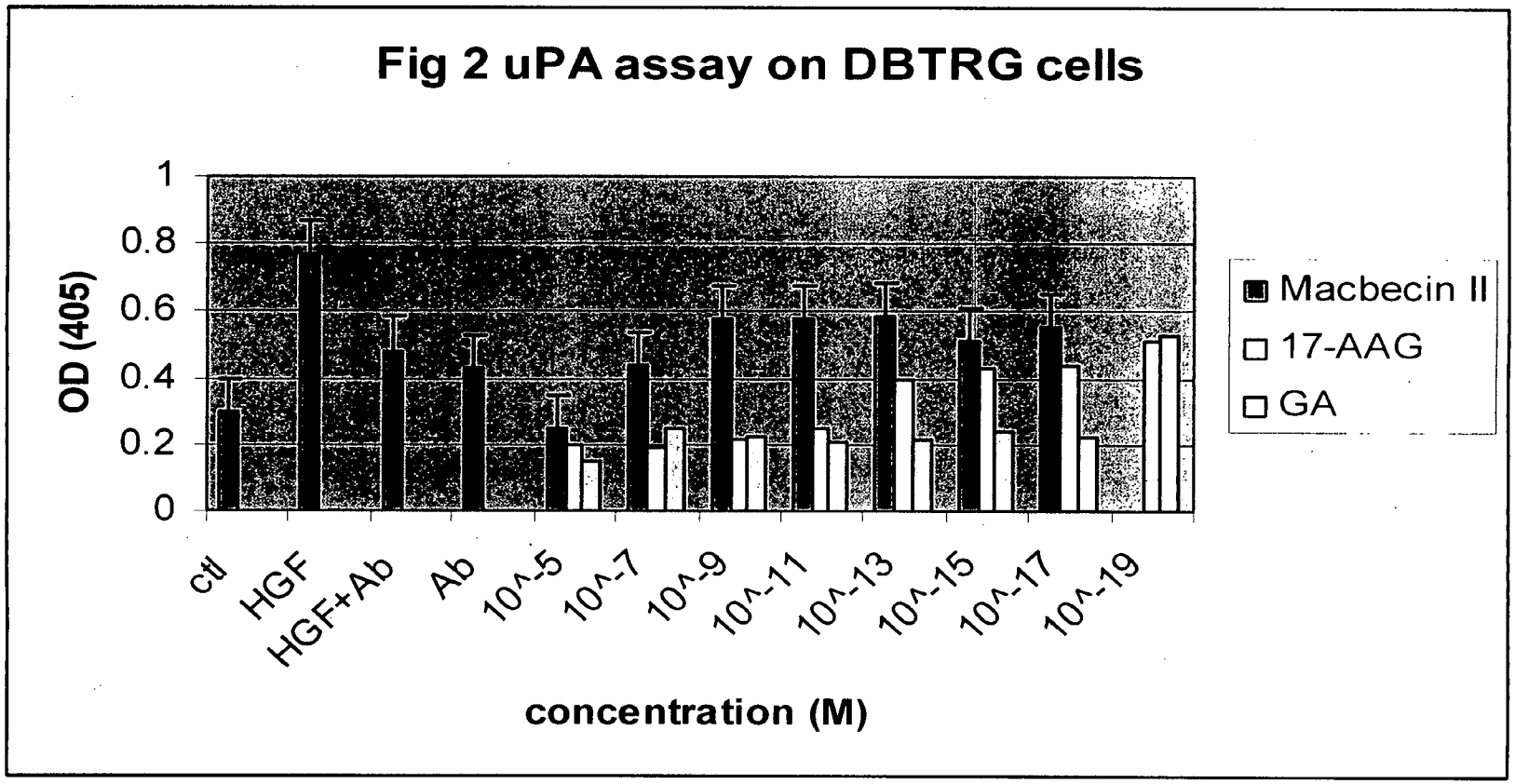
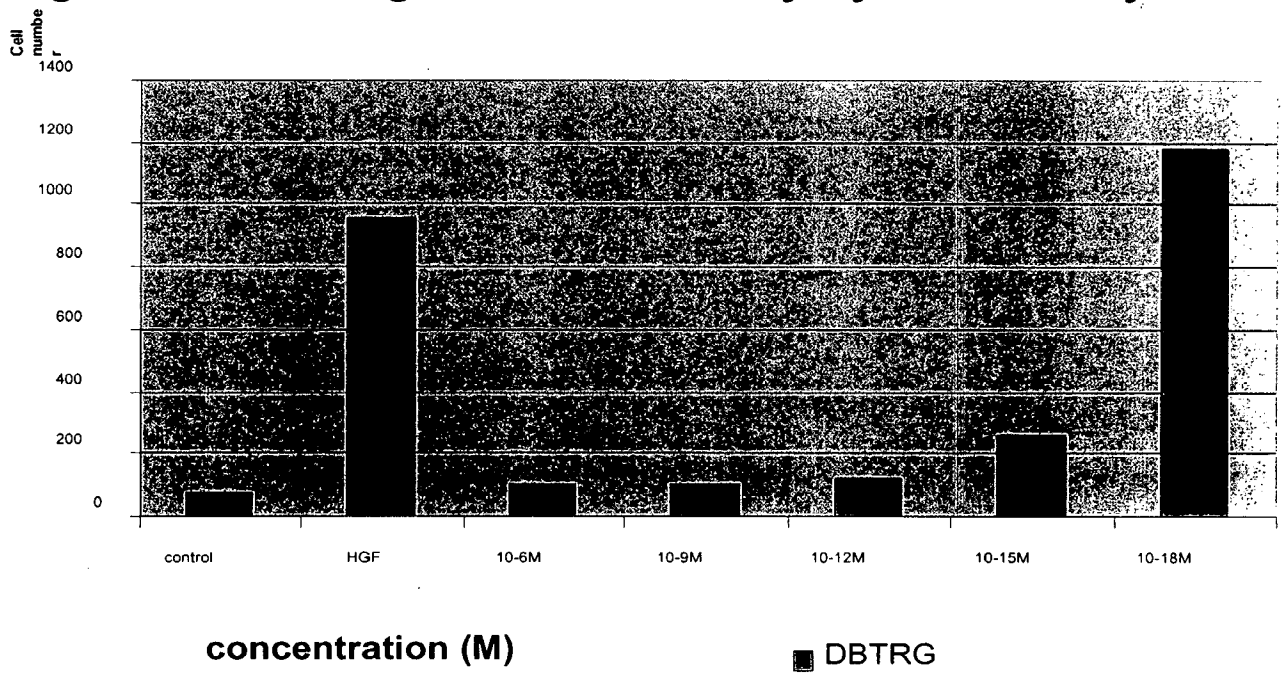


Fig 2 uPA assay on DBTRG cells
OD (405)
1
0.8
0.6
0.4
0.2
0
ctl
HGF
HGF+Ab
Ab
10^-5
10^-7
10^-9
10^-11
10^-13
10^-15
10^-17
10^-19
concentration (M)
Macbecin II
17-AAG
GA

Figure 3. Matrigel invasion assay by Geldanamycin



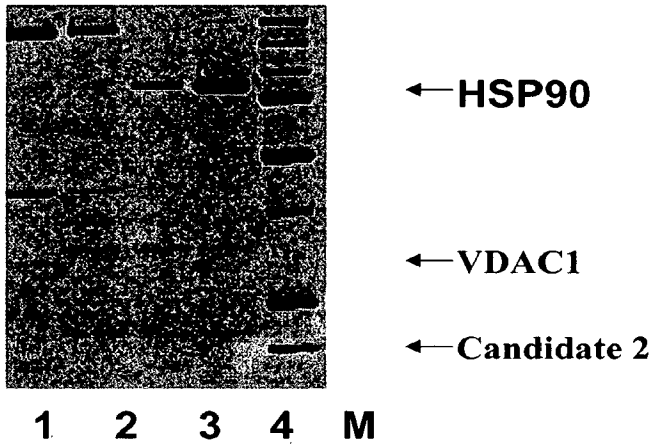


Fig 4. Geldanamycin (GA) pull-down assays

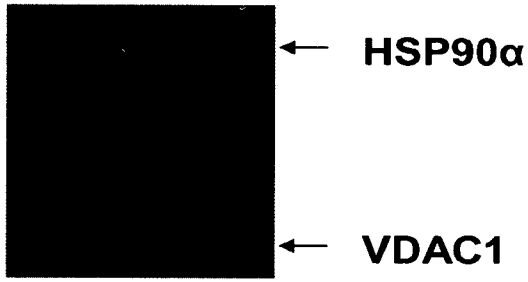


Figure 5 GA pull-down of porin from mitochondrial enrichment

APPLICATION DATA SHEET

Application Information

Application Number::	
Filing Date::	
Application Type::	Provisional
Subject Matter::	Utility
Suggested Classification::	
Suggested Group Art Unit::	
CD-ROM or CD-R?::	
Number of CD Disks::	
Number of Copies of CDs::	
Sequence Submission?::	
Computer Readable Form (CFR)?::	
Number of Copies of CFR::	
Title::	GELDANAMYCIN AND DERIVATIVES INHIBIT CANCER INVASION AND IDENTIFY NOVEL TARGETS
Attorney Docket Number::	38345-201708
Request for Early Publication?::	NO
Request for Non-Publication?::	NO
Suggested Drawing Figure::	
Total Drawing Sheets::	5
Small Entity?::	YES
Latin Name::	
Variety Denomination Name::	
Petition Included?::	
Petition Type::	
Licensed US Govt. Agency::	
Contract or Grant Numbers::	
Secrecy Order in Parent Appl.::	

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